

## **RECOMBINANT CALF-CHYMOSIN AND A PROCESS FOR PRODUCING THE SAME**

### **FIELD OF THE INVENTION:**

The present invention relates to recombinant calf-chymosin and a process for producing the same.

### **BACKGROUND OF THE INVENTION:**

Chymosin is an enzyme which is particularly useful in the preparation of cheese. Natural sources of chymosin include stomachs of calf, goat, lamb, porcine and the like. However, commercial chymosin is primarily obtained from the fourth stomach of milk fed calves. Alternate sources of chymosin have been developed particularly because of the decrease in calf production. Production and extraction of commercially valuable proteins from recombinant microorganisms encouraged the study of producing and purifying microbially produced chymosin. However, a process suitable for commercial scale production and recovery of chymosin has not been developed so far.

### **OBJECTS OF THE INVENTION:**

The main object of the present invention is to produce recombinant calf-chymosin, an enzyme particularly useful in the preparation of cheese.

Yet another main object of the present invention is to isolate the chymosin gene from calf tissues.

Still another main object of the present invention is to clone the chymosin gene in a bacterial expression vector.

Still another main object of the present invention is to express the recombinant calf-chymosin in *E.coli* cell.

Still another main object of the present invention is to provide an efficient process for expressing prochymosin gene and its conversion into enzymatically active pure chymosin.

#### **STATEMENT OF THE PRESENT INVENTION:**

The present invention relates to a recombinant calf-Chymosin protein as set forth in SEQ ID No. 1; a recombinant calf-Chymosin gene as set forth in SEQ ID No. 2 encoding the protein comprising amino acid sequence of SEQ ID NO.1; an *E.coli* comprising the recombinant calf-chymosin gene of SEQ ID No. 2; an expression vector pET21b comprising recombinant calf-chymosin gene as set forth in SEQ ID No. 2; and lastly a method for producing recombinant calf-chymosin protein as set forth in SEQ ID No. 1 which comprises steps of isolating calf-chymosin gene, cloning the same in bacterial expression vector pET21b, transforming said cloned vector into cells of *E.coli*, fermenting said *E.coli* to produce pro-chymosin, converting said pro-chymosin to chymosin and subsequently recovering the recombinant calf-chymosin.

#### **DETAILED DESCRIPTION OF THE INVENTION:**

The present invention relates to a recombinant calf-Chymosin protein as set forth in SEQ ID No. 1

In yet another embodiment of the present invention, the protein comprising amino acid sequence of SEQ ID No.1 is encoded by a recombinant calf-chymosin gene as set forth in SEQ ID No.2

In still another embodiment of the present invention, an *E.coli* comprising the recombinant calf-chymosin gene of SEQ ID No. 2.

In still another embodiment of the present invention *E. coli* cells are BL21 cells.

In still another embodiment of the present invention, an expression vector pET21b comprising recombinant calf-chymosin gene as set forth in SEQ ID No. 2.

In still another embodiment of the present invention a method for producing recombinant calf-chymosin protein as set forth in SEQ ID No. 1 which comprises steps of isolating calf-chymosin gene, cloning the same in bacterial expression vector pET21b, transforming said cloned vector into cells of *E.coli*, fermenting said *E.coli* to produce pro-chymosin, converting said pro-chymosin to chymosin and subsequently recovering the recombinant calf-chymosin.

In still another embodiment of the present invention, the calf-chymosin gene is obtained by isolating RNA from fourth stomach of calf tissue, synthesising a first strand of cDNA therefrom by treating the same with a reverse primer of SEQ ID NO.3 and then with a forward primer of SEQ ID NO.4.

In still another embodiment of the present invention the cDNA is ligated at small site of pBSSK+ plasmid and then transformed into TOP10 cells of *E. coli*.

In still another embodiment of the present invention recombinant clones were identified and treated with a forward primer of SEQ ID NO.5 and reverse primer of SEQ ID NO.6 containing Nde I and Hind III sites to obtain an amplified fragment.

In still another embodiment of the present invention the amplified fragment is transformed into cells of *E.coli* for expressing chymosin gene.

In still another embodiment of the present invention *E.coli* cells containing recombinant calf-chymosin gene is fermented, the suspended cells produced on completion of fermentation are lysed, chilled and pH adjusted to about 8 before incubation at room temperature and the separation of supernatant containing prochymosin.

In still another embodiment of the present invention the pH of supernatant is adjusted to about 2 for activation, further incubated for about 6 hrs and subjected to filtration to obtain filtrate.

In still another embodiment of the present invention the filtrate is subjected to sodium chloride precipitation, then the precipitate is dissolved followed by the addition of sodium benzoate as preservative.

In the instant invention, Calf-chymosin gene is isolated preferably from the fourth stomach of milk fed calf tissues. Recombinant calf-chymosin is produced by cloning chymosin gene with bacterial expression vector pET21b and is transformed into E-coli strain. This E-coli strain containing recombinant calf-chymosin gene is fermented under suitable conditions preferably in a culture medium developed by us. This medium contains the following

Peptone - 12g/l

Yeast Extract - 24g/l

Sodium chloride --- 10g/l

Prochymosin produced during fermentation is subjected to denaturation by increasing the pH of the medium to 10-11. The suspension then diluted and the pH reduced to about 8 for effective renaturation of the protein. The prochymosin thus obtained is then acidified for activation and is further processed.

This invention relates to a process for producing recombinant calf-chymosin which comprises the steps of isolating calf-chymosin gene, cloning the same in bacterial expression vector pET21b, transforming said cloned vector into cells of E- coli, fermenting said E-coli strains to produce pro-chymosin, converting said pro-chymosin to chymosin and subsequently recovering the recombinant calf-chymosin. This invention also includes recombinant calf-chymosin having the following aminoacid sequence as setforth in SEQ ID No. 1 and the corresponding gene sequence as setforth in SEQ ID No. 2.

**SEQ ID No. 1. Recombinant Calf-Chymosin Protein Sequence**

Met A S I T R I P L Y K G K S L R K A L K E H G L L E D F L Q K Q Q Y G I S S K  
Y S G F G E V A S V P L T N Y L D S Q Y F G K I Y L G T P P Q E F T V L F D T G  
S S D F W V P S I Y C K S N A C K N H Q R F D P R K S S T F Q N L G K P L S I H  
Y G T G S M Q G I L G Y D T V T V S N I V D I Q Q T G G L S T Q E P G D V F T Y  
A E F D G I L G M A Y P S L A S E Y S I P V F D N M M N R H L V A Q D L F S V  
Y M D R N G Q E S M L T L G A I D P S Y Y T G S L H W V P V T V Q Q Y W Q F  
T V D S V T I S G V V V A C E G G C Q A I L D T G T S K L V G P S S D I L N I Q  
Q A I G A T Q N Q Y D E F D I D C N N L S Y M P T V V F E I N G K M Y P L T P S  
A Y T S Q D Q G F C T S G F Q S E N H S Q K W I L W D V F I R E Y Y S V F D R  
A N N L V G L A K A I Stop

**SEQ ID No. 2. Recombinant Calf-Chymosin Gene Sequence**

A T G G C T A G C A T C A C T A G G A T C C C T C T G T A C A A A G G C A A G T C T C T G  
A G G A A G G C G C T G A A G G A G C A T G G G C T T C T G G A G G A C T T C C T G C A G  
A A A C A G C A G T A T G G C A T C A G C A G C A A G T A C T C C G G C T T C G G G G A G  
G T G G C C A G C G T G C C C C T G A C C A A C T A C C T G G A T A G T C A G T A C T T T  
G G G A A G A T C T A C C T C G G G A C C C G C C C C A G G A G T T C A C C G T G C T G  
T T T G A C A C T G G C T C C T C T G A C T T C T G G G T A C C C T C T A T C T A C T G C A A G  
A G C A A T G C C T G C A A A A A C C A C C A G C G C T T C G A C C C G A G A A G T C G  
T C C A C C T T C C A G A A C C T G G G C A A G C C C C T G T C T A T C A C T A C G G G  
A C A G G C A G C A T G C A G G G C A T C C T G G G C T A T G A C A C C G T C A C T G T C  
T C C A A C A T T G T G G A C A T C C A G C A G A C A G G A G G C C T G A G C A C C C A G  
G A G C C C G G G G A C G T C T T C A C C T A T G C C G A A T T C G A C G G G A T C C T G  
G G G A T G G C C T A C C C C T C G C T C G C C T C A G A G T A C T C G A T A C C C G T G  
T T T G A C A A C A T G A T G A A C A G G C A C C T G G T G G C C A A G A C C T G T T C  
T C G G T T T A C A T G G A C A G G A A T G G C C A G G A G A G C A T G C T C A C G T T G  
G G G G C C A T C G A C C C G T C C T A C T A C A C A G G G T C C C T G C A C T G G G T G  
C C C G T G A C A G T G C A G C A G T A C T G G C A G T T C A C T G T G G A C A G T G T C

ACC ATC AGC GGT GTG GTT GTG GCC TGT GAG GGT GGC TGT CAG GCC  
 ATC CTG GAC ACG GGC ACC TCC AAG CTG GTC GGG CCC AGC AGC GAC  
 ATC CTC AAC ATC CAG CAG GCC ATT GGA GCC ACA CAG AAC CAG TAC  
 GAT GAG TTT GAC ATC GAC TGC AAC AAC CTG AGC TAC ATG CCC ACT  
 GTG GTC TTT GAG ATC AAT GGC AAA ATG TAC CCA CTG ACC CCC TCC  
 GCC TAT ACC AGC CAG GAC CAG GGC TTC TGT ACC AGT GGC TTC CAG  
 AGT GAA AAT CAT TCC CAG AAA TGG ATC CTG TGG GAT GTT TTC ATC  
 CGA GAG TAT TAC AGC GTC TTT GAC AGG GCC AAC AAC CTC GTG GGG  
 CTG GCC AAA GCC ATC TGA

In the above sequence, amino acids shown in red indicate sequence variation of chymosin gene of our invention compared to the reported and published sequence.

A recombinant calf-Chymosin protein is set forth in SEQ ID No. 1, wherein the replacement of single amino acid Aspartic Acid (D) with Glycine (G) at position 287 is also covered and is referred to as SEQ ID No.1.

A recombinant calf-Chymosin gene is set forth in SEQ ID No. 2, wherein the replacement of nucleotide GAT with GCC at position 287 is also covered and is referred to as SEQ ID No.2.

The invention is further elaborated with the help of following examples. However, these examples should not be construed to limit the scope of the invention.

#### **EXAMPLES:**

##### **Example 1: Isolation of calf-chymosin gene**

Total RNA was isolated from the 4<sup>th</sup> stomach of calf tissue. The tissue was frozen and ground to a fine powder. The powder was transferred to a 50ml centrifuge tube containing 10ml of denaturing buffer (4M Guanidine thiocyanate, 25mM Sodium citrate pH 7.0, 0.5% Sarkosyl and 0.1M 2-Mercaptoethanol). To this 1ml of 2M Sodium acetate pH 4.0, 10ml of Phenol and 2ml of Chloroform were added and kept on ice for 20 min.

Later, it was centrifuged for 15 min at 12,000rpm, 4°C. The upper aqueous phase was transferred carefully into a new tube to which an equal volume of isopropanol was added and kept at -20°C for 1 hr. The RNA was precipitated at 15,000rpm for 15min. at 4°C. The RNA pellet was washed with 70% ethanol and dissolved in 1ml of denaturing buffer followed by two successive extractions with phenol : chloroform : isoamyl alcohol (30 : 29 : 1). The RNA was precipitated with 1/10 volume of 3M sodium acetate (pH 5.2) and 2.5 volume of ethanol and dissolved in 500µl of DEPC treated water.

For the synthesis of first strand of the cDNA, 10µg of RNA was dissolved in 16µl DEPC water and the following components were added: 2µl of 10mM dNTP mix, 2µl of 1µg of reverse primer (5'-TGT GGG GAC AGT GAG GTT CTT GGT C- 3'), 4µl of 10X RT buffer (200mM Tris-HCl, pH 8.4, 500mM KCl), 8µl of 25mM MgCl<sub>2</sub>, 4µl of 0.1M DTT, 2µl of Rnase inhibitor, 2µl (50 units) of Superscript II reverse transcriptase. The mixture was incubated for 50min at 42°C and the reaction is stopped by inactivating the enzyme at 70°C for 15 min. PCR amplification of prepro chymosin was performed using the 50ng of 1st strand cDNA with a reverse primer (5'-TGT GGG GAC AGT GAG GTT CTT GGT C-3'), and a forward primer (5'-ATG AGG TGT CTC GTG GTG CTA CTT-3') in a thermal cycler programmed as (step 1 : 95°C-5'; step 2: 94°C-30sec; step 3: 54°C-30sec; step 4: 72°C-1min; step 5: go to step 2 34 times; step 6: 72°C-7min; step 7: end). The PCR reaction when analyzed on 1.0% agarose gel showed an amplified band of 1.2kb. The 1.2kb fragment was cut with a sterile blade and the gel slice was dissolved in 500µl of Tris saturated phenol and left in liquid nitrogen for a few min. The microcentrifuge tube was allowed to come to room temperature and centrifuged for 5min at 12,000rpm, 4°C. The upper aqueous phase was extracted with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and DNA was precipitated with 1/10th volume sodium acetate and 2.5 volume ethanol at -70°C for 1h. DNA was precipitated at 15,000rpm for 15 min. The pellet was dried and dissolved in sterile distilled water. This eluted 1.2kb fragment was ligated at SmaI site of pBSSK+ plasmid, which was then transformed in to TOP10 cells of E.coli. The recombinant clones were selected (blue white screening) and checked with restriction digestion analysis of the plasmids. Recombinant plasmid was taken as a template and a PCR was performed using a forward primer (5'-GAT ATA CAT ATG

GCT AGC ATC ACT AGG ATC CCT CTG TAC-3') and reverse primer (5'-GCA GTA AGC TTG ACA GTG AGG TTC TTG GTC AGC G-3') containing Nde I and Hind III sites. An amplified band of 1098bp was observed when the PCR product was analyzed on 1.0% agarose gel. This amplified fragment of 1098bp was eluted from the gel and ligated in pET21b expression vector at Nde I and Hind III sites and transformed in to BL21 cells of E.coli for the expression of the chymosin gene.

#### **Example 2: Fermentation of recombinant E.coli expressing calf-chymosin**

Fermentation of E.coli cells containing recombinant calf-chymosin gene was carried out in 15 L fermentor with 6 L of working volume. Fermentation was carried out in SBL medium as herein after described using 4% inoculum as seed. The fermentation process lasts for 22-24 h., and the whole procedure can be divided into four stages:

Stage 1 : Preparation of SBL media.

Stage 2 : Preparation of Accessories for fermentation.

Stage 3 : Preparation of Seed.

Stage 4 : Process of Fermentation.

Composition of the SBL medium:

Peptone : 12g/L

Yeast Extract : . 24g/L

Sodium Chloride : 10g/L

These ingredients were calculated for 6 L, weighed and dissolved in 5.75 L of distilled water and later volume was made upto 6L with water, pH was set to 7.0 using 4N NaOH. 240 ml was separated out from 6L and was autoclaved separately at 15 lb/sq.inch for 20 min. The remaining 5.76 L was taken in fermentor vessel, 1ml of sigma A concentrate antifoam was added and autoclaved with the vessel at 15 lb/sq.inch for 45 min.



**Example 3: Preparation of supplements for fermentation:**

1. Lactose: 0.4 % (w/v) lactose for 6L medium was prepared by dissolving 24g in 150ml distilled water, autoclaved at 15 lb/sq.inch for 20 min. 0.2% (w/v) lactose for 6L medium was prepared by dissolving 12g in 150ml distilled water, autoclaved at 15 lb/sq.inch for 20 min.
2. Glycerol: 0.3% (v/v) Glycerol for 6 L medium was made by adding 18ml of glycerol to 82 ml of water, autoclaved at 15 lb/sq.inch for 20 min.
3. Ampicillin: 100 mg/ml ampicillin was made by dissolving 1200mg of sodium salt of ampicillin in 12ml of distilled water. The solution was filter sterilized by passing through 0.2 microns millipore syringe filter.
4. IPTG (Isopropyl- $\beta$ -D-thiogalactopyranoside): 2mM IPTG (for 6L medium) was prepared by dissolving 2.85g of IPTG in 20ml distilled water. The solution was filter sterilized by passing through 0.2 microns millipore syringe filter.
5. 4N NaOH: 100 ml of 4N NaOH was made by dissolving 16g of NaOH pellets in 90ml of autoclaved water, and after dissolution, volume is made upto 100ml with water.

**Example 4: Preparation of seed:**

4% SBL medium (240 ml of 6L medium) was inoculated with 100 $\mu$ l glycerol stock of E.coli cells in 500ml baffled flask containing 100 $\mu$ g/ml ampicillin. The flask was kept shaking at 37°C, 250rpm for 18 h. Optical Density (OD) of the culture was read at 600nm in Shimadzu Spectrophotometer.

#### **Example 5: Fermentation process:**

The whole process of fermentation begins with inoculation of seed at 4% into SBL medium. The pre-grown seed is inoculated into 5.76 L autoclaved SBL medium. Along with the seed -0.3% glycerol, 6ml of 100µg/ml ampicillin were also added through the inlet pump. Prior to addition of seed, the fermentor was made ready by calibrating different probes like pH probe, DO probe and Temperature probe.

- a) pH probe calibration: pH probe was calibrated using standard pH 4.0 and pH 7.0 solutions.
- b) Dissolved Oxygen (DO) probe calibration: DO probe was calibrated by using 5% sodium nitrite solution for 0% DO.
- c) Temperature probe calibration: Temperature probe was checked using water at different temperatures in a standard water bath.

**Fermentation conditions:** The fermentation parameters set were given as below, and the fermentation was started by quick addition of the seed into the inoculation port.

Temperature	:	37°C,
pH	:	7.0,
Agitation	:	350 rpm,
Dissolved Oxygen	:	30 %
SLPM	:	1.25
VVM	:	0.2

After 2h of inoculation 6ml of 100µg/ml ampicillin was added. When OD (at 600nm) reaches about 4-5 (after 3h.) 0.4 % Lactose was added. Then the temperature was reduced to 32°C. When OD reaches 7-8 (after 5h.) 0.2 % Lactose was added. Agitation speed was

then increased to 450 rpm. When the OD (at 600nm) reaches 10.0, the culture batch was induced with 2mM IPTG.

**pH monitoring during the fermentation process:** pH was monitored carefully during the process of fermentation from the seed inoculation stage till the end. Initially during the growth phase of bacteria the pH of the culture drops, and the pH is maintained at 7.0 using 4N NaOH. After substantial growth of bacteria, pH shoots above 7.0 and addition of 4N NaOH was completely stopped. Samples of 1ml were collected from the fermentor at different time points, viz., uninduced (immediately before IPTG addition), 3h., 6h., and 9h, after IPTG addition, and were processed for loading onto the gel for SDS-PAGE analysis.

**Cell harvesting:** After running the fermentor for 20 to 24 h following seed inoculation, and when the OD (at 600 nm) of the fermentor sample reads to ~ 20 O.D./ml, the fermentor batch was terminated by switching off all the controls.

**Pelleting and storage of the cells:** After termination of the batch, the cell culture was pelleted by centrifuging at 8000x g for 10 min. Supernatant was discarded and the pellet was stored in -70°C freezer until further use.

**Extraction and purification of chymosin enzyme:**

E.coli cells after fermentation were suspended in 3.5 to 4 volumes of 10mM EDTA (pre-chilled, 4°C) and the suspension was incubated at 4°C for 30 to 60 min to obtain homogeneous suspension. Later the suspension was subjected to lysis by adding equal volume of alkali solution (0.2 N Sodium hydroxide) to a final concentration of 0.1 N with continuous stirring for 15-20min at 4°C. For complete denaturation and effective renaturation in the subsequent step, the lysed suspension was diluted to 9-12 folds with pre-chilled (4°C) aqueous solution (H<sub>2</sub>O). Diluted suspension was allowed to stand at 4°C for 30 min and the pH was readjusted to 8.0 by addition of 1.0 M glycine solution to a final concentration of 56 mM and allowed to stand at 4°C for 30 min. The inactive form-pro-chymosin at this stage was incubated for 72 h at RT (28 ±2°C) for proper refolding.

During this incubation period, the cell debris and other solid masses (Nucleic acid complexes) settles to the bottom and the supernatant can be decanted to obtain clear folded pro-chymosin.

In the subsequent steps the pH of pro-chymosin was adjusted to 2.0 for the activation. The adjustment is mainly by addition of a buffer with pH 1.5 (1.0M Hydrochloric acid and 1.0M glycine in 0.8: 1.0-ratio). The extract was kept at low pH for a period of 6-8 h. Following the above activation, the crude low pH extract was subjected to a step where by precipitated impurities can be separated. This separation can be achieved by conventional industrial separation methods such as filtration (Whatmann No.3). Hence, the process is economically efficient, can be easily scaled up for commercial production.

The supernatant or filtrate resulting from the above separation containing the extracted milk clotting enzyme can be processed in any one of the three methods. In method I the enzyme was concentrated by subjecting to sodium chloride precipitation to about 5.8-6.0 M. Precipitation was usually carried at 4°C by gradual addition of the required amount of salt and subjecting to continuous stirring for an hour after complete addition of the salt. The solution was then subjected to vacuum filtration using 0.2 micron nylon membranes and the supernatant free of any enzyme can be discarded. The wet precipitate was resuspended in a 4.0 pH buffer and subsequently increased to pH 5.0 and formulated for stability.

In Method II, the pH of the filtrate was increased to the pH (to 4.7; or to 5.0) and the same was maintained at 32°C for about an hour and subjected to another filtration to obtain clear chymosin. However, the higher pH ranges around 5.0 are not preferred, which may reduce the stability of the enzyme during storage might be due to the exposure of the active site during the process. Hence, the preferred method of activation is the acidification followed by precipitation.

In Method III, after acidification step the pH of the filtrate was increased to 4.7 and subjected to sodium chloride precipitation. The precipitate was dissolved as mentioned in Method I. The chymosin produced in all the above three methods is substantially pure,

needs to be formulated to the desired specifications for final use. The salt concentration for formulation (NaCl) was brought to about 10% and a preservative such as Sodium benzoate was added. The enzymatic strengths were measured in terms of IMCU (International Milk Clotting Units). In the present investigation, 6000-9000 IMCU per gram of biomass was obtained.

Solutions and reagents required for chymosin process:

Solution A : 0.01M EDTA

Solution B : 0.2N Sodium hydroxide

Solution C : Autoclaved water

Solution D : 1.0M Glycine

Solution E : I) Solution D

II) 1.0N Hydrochloric acid

Mix (I) and (II) in 1 : 0.8 ratio and pH should be 1.5

Solution F : 5.0M Sodium chloride

Solution G : I) Solution D

II) 0.5N Sodium hydroxide

Mix (I) and (II) in 1 : 0.4 ratio and pH should be 9.5

Reagent H : Sodium benzoate

Reagent I : Sodium chloride

Solution J : 0.2M Glycine with 0.001M EDTA.

**Solution J :** Adjust the pH of solution J to 4.0 with Solution E .

**Solution J :** Adjust the pH of solution J to 5.0 with Solution E.

**Reagent K :** Trehalose-filter sterilized solution (10%).